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Serum Esterases

2. AN ENZYME HYDROLYSING DIETHYL *p*-NITROPHENYL PHOSPHATE (E 600) AND ITS IDENTITY WITH THE A-ESTERASE OF MAMMALIAN SERA

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It has been stated (Roche, 1950): 'The orthophosphoric tri-esters which are easily hydrolysed by acids, bases or even boiling water, are not hydrolysed enzymatically.' If a solution of diethyl *p*-nitrophenyl phosphate (E 600) is added to rabbit serum the serum rapidly turns yellow. This is due to the liberation of *p*-nitrophenol by the hydrolysis of E 600.

E 600 is an orthophosphoric tri-ester and has been studied primarily for its anticholinesterase properties. It is a very active inhibitor (Aldridge, 1950; Aldridge & Davison, 1952) and will inhibit the cholinesterase activity of sheep erythrocytes by 50% after incubation at 37° for 30 min. at a concentration of 2×10^{-8} M. It is also very toxic, the LD_{50} for rats being about 0·5 mg./kg. It is therefore of interest that this substance can be hydrolysed in mammals. In this paper, methods for the determination of this enzyme (E 600-esterase) are given followed by a study of its properties and distribution in some species.

It has been shown (Aldridge, 1953) that there are two distinct types of esterase which will hydrolyse *p*-nitrophenyl acetate, propionate and butyrate. One esterase (A-esterase) is not inhibited by E 600 while the other (B-esterase) is inhibited by concentrations as low as 10^{-8} M. In this paper evidence will be presented which shows that the enzyme hydrolysing E 600 and A-esterase are identical enzymes. A preliminary report of these observations has been published (Aldridge, 1951).

The work described in this paper is part of a thesis which has been accepted by the University of London for the degree (external) of Doctor of Philosophy in the Faculty of Science.

EXPERIMENTAL

Materials

Diethyl *p*-nitrophenyl phosphate was first prepared by Schrader (1947) who called it E 600. It is a pale yellow liquid, sp.gr. 1·27, practically non-volatile at room temperature and atmospheric pressure, b.p. 160° at 0·05 mm. Hg pressure. It is soluble in water at 25–37° to 2·37 mg./ml. and is slowly hydrolysed in buffers at pH 7·8 to give diethyl phosphoric acid and *p*-nitrophenol, no orthophosphate being produced. The first-order constant for its hydrolysis at 37° in Sørensen's M/15 phosphate buffer, pH 7·6, is $3 \cdot 9 \times 10^{-5}$ (min.$^{-1}$), the half life under these conditions being 11·2 days. I am grateful to Mr B. Topley (Albright and Wilson Ltd.) for a generous supply of E 600.

Manometric method of esterase estimation

Since two molecules of acid are liberated for every molecule of E600 hydrolysed it is possible to use a method similar to that developed by Ammon (1933) for the determination of cholinesterase.

Reagents. (*a*) Buffer. $NaHCO_3$, 0·031 M; NaCl, 0·162 M; gelatin, 0·1% (w/v). When gassed with 5% (v/v) CO_2 in 95% N_2 a solution of pH 7·73 at 37° is obtained. (*b*) Buffer-substrate. 4 drops E600 (approx. 80 mg.) are dissolved in 0·5 ml. methanol and then blown rapidly from a Pasteur pipette into 25 ml. buffer.

Procedure. The output of CO_2 is determined after tipping 0·5 ml. of a suitable dilution of enzyme into 3·5 ml. buffer-substrate. The results are calculated using the simplified linear regression method of Aldridge, Berry & Davies (1949). A linear output of CO_2 with respect to time and the output of CO_2/min. is a linear function of the enzyme concentration. Without the addition of gelatin there is a slight loss of activity during the determination. The determination of A-esterase is similar to the above and has been described in detail (Aldridge, 1953).

Colorimetric method

p-Nitrophenol liberated during the hydrolysis of E600 has an intense yellow colour at pH 7 or above. A colorimetric method has been developed for use in the routine determination of the enzyme distribution in tissues. *p*-Nitrophenol has been extracted from the highly coloured and turbid suspensions with a 1:1 (v/v) mixture of *n*-butanol and toluene.

Reagents. (*a*) Sørensen's M/15 phosphate buffer, pH 7·6. (*b*) E600, which should be free from uncombined *p*-nitrophenol. An approximately 10% (v/v) solution of E600 in $CHCl_3$ is washed with equal volumes of 2% (w/v) $NaHCO_3$ until only a very pale yellow aqueous layer is obtained. All the bicarbonate washings are united and washed with 15 ml. $CHCl_3$. All the $CHCl_3$ washings are united and washed once with water and once with 1% (w/v) citric acid solution. The $CHCl_3$ is run through a filter paper, dried with anhydrous $CaSO_4$ and the $CHCl_3$ evaporated in a stream of dry air. (*c*) Buffer-substrate. One drop (about 20 mg.) of purified E600 is added to 10 ml. buffer. It is shaken vigorously. (*d*) Butanol/toluene mixture. Equal volumes are mixed. (*e*) Ammonia (sp.gr. 0·880) and absolute ethanol (1:4, v/v). (*f*) Hydrochloric acid, approx. 5 N. (*g*) Gelatin 2% (w/v).

Procedure. Into two tubes *A* and *B* 9·5 ml. of buffer-substrate are pipetted and warmed to 37°. In a separate tube, serum or tissue homogenate suitably diluted with 2% (w/v) gelatin are also warmed. To tube *A* 0·5 ml. of enzyme preparation is added, mixed and left at 37°. After 30 min. 0·5 ml. of 5 N-HCl is added to both tubes followed by 0·5 ml. of enzyme preparation to tube *B*. To each, 10 ml. of butanol/toluene mixture are added and shaken vigorously. After separation has taken place not more than 5 ml. of the solvent layer are pipetted off. Just before reading at 420 mμ. 3 ml. of 20% (v/v) ammonia in ethanol are added and the volume adjusted to 10 ml. with butanol/toluene mixture. The *p*-nitrophenol present is read off from a calibration curve prepared in a similar way. $A - B = \mu g.$ *p*-nitrophenol released by 0·5 ml. of enzyme preparation in 30 min. The activity of the preparation is expressed as mg. *p*-nitrophenol liberated in 30 min. at 37° by 1 g. or 1 ml. of tissue. Under these conditions the amount of *p*-nitrophenol liberated is a linear function of enzyme concentration.

Correlation between manometric and colorimetric methods. During the hydrolysis of E600, 322 μl. CO_2 liberated in the manometric method are equivalent to 1 mg. *p*-nitrophenol. Therefore mg. *p*-nitrophenol/g./30 min. $\times$ 10·7 $\equiv$ μl. CO_2/ml./min.

A-esterase free from B-esterase and cholinesterase activities may be prepared by incubation with 5 μg./ml. E600 for 30 min. at 37°. Under these conditions both B-esterase and the cholinesterases are completely inhibited.

RESULTS

E600-esterase

Enzymic nature of hydrolysis of E600. The hydrolysis of E600 by rabbit serum is continuous. The rate of this hydrolysis is much higher than the aqueous hydrolysis of E600 and is proportional to the amount of serum taken. Heating for 5 min. at 68° completely prevents the catalysis of the hydrolysis of E600 while after 5 min. at 50° it is slightly affected. After dialysis for 2 days at 40° against phosphate buffer, pH 7·6, the solution, although considerably diluted, showed the same activity/mg. of protein as the original preparation. It was concluded that this hydrolysis was an enzymic process. Throughout this paper the enzyme will be called E600-esterase for, as will be shown later, the enzyme, although it hydrolyses a tri-ester of phosphoric acid, is not related to the phosphatases and has the properties of an esterase.

Table 1. pH *optima of E600-esterase in phosphate buffer*

(Enzyme preparation; 0·5 ml. of 100-fold diluted rabbit serum, M/15 Sørensen phosphate buffers of various pH values. Activity determined by the colorimetric method.)

pH	*p*-Nitrophenol liberated in 30 min. (μg.)
8·2	56
8·0	72
7·8	82
7·6	85
7·4	87
7·2	73
7·0	61
6·8	33
6·6	24

Since rabbit serum is a particularly potent and convenient source of the enzyme most of the work to follow has been carried out using this preparation. In Table 1 is shown the effect of varying the pH on the activity of the enzyme. The pH optimum is between 7·4 and 7·6 and appears to be the same for phosphate, borax-boric acid, veronal-acetate and bicarbonate-carbon dioxide buffering systems.

However, veronal-acetate appears to lower the activity roughly 35 %. The variation of the rate of hydrolysis with different concentrations of substrate is given in Fig. 1. The results have been plotted according to the procedure of Lineweaver & Burk (1934) and thus evaluated K_m is $4{\cdot}5 \times 10^{-4}$ M. This value indicates a fairly high affinity of E 600-esterase for its substrate. The sensitivity of E 600-esterase to the inhibitors nickel sulphate, copper sulphate and *p*-chloromercuribenzoic acid has been determined and the results are shown in Table 2. It is clear that this enzyme is extremely sensitive to these inhibitors. This inhibition by nickel and *p*-chloromercuribenzoic acid is reversed by glutathione and cysteine. E 600-esterase is only inhibited 27 % after incubation with 9×10^{-3} M-sodium iodoacetate

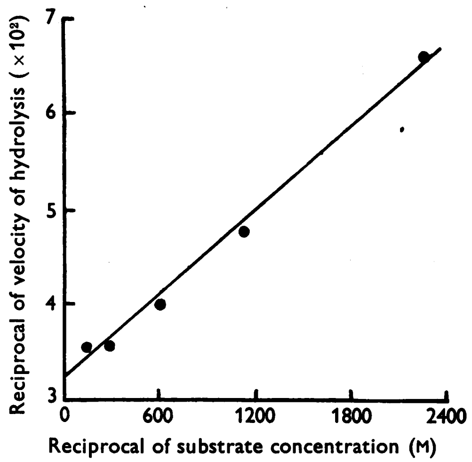

Fig. 1. The relation between the concentration of E 600 and its hydrolysis by rabbit serum. The 'initial velocity' of hydrolysis is given as μl. CO_2 liberated during the first 10 min. The value calculated for K_m from these results is $4{\cdot}5 \times 10^{-4}$ M.

for 30 min. at 37°. Iodoacetate is known to vary in its action on —SH groups (Dixon, 1948). The evidence is therefore suggestive, but by no means conclusive, that E 600-esterase requires —SH groups for its activity. Eserine, which is the best available inhibitor to differentiate ali-esterase from the cholinesterases (Richter & Croft, 1942; Myers & Mendel, 1949), has practically no effect on E 600-esterase, causing only 8 % inhibition at 8×10^{-4} M.

Table 3 shows the results of a crude fractionation of rabbit serum by the procedure of Cohn *et al.* (1950) at −5° and by ammonium sulphate at +5°. These two sets of results are in striking contrast for, whereas using Cohn's method the enzyme proteins came down in the globulin fraction, using ammonium sulphate E 600-esterase is still mostly in solution even in 80 % saturated solutions, i.e. the albumin fraction. The possibility must be considered, and particularly with Cohn's method, that protein interactions between various components of the mixture have caused precipitation at unexpected stages of the separation.

Distribution of E 600-esterase in tissues. Aromatic nitro compounds are reduced by enzyme systems to amino compounds (Williams, 1947; Bueding & Joliffe, 1946; Westfall, 1943; Smith & Worrell, 1950; Egami, Ebata & Sato, 1951; Parker, 1952). Such reductions are prevented by the addition of cyanide (Egami *et al.* 1951; Parker, 1952). There are, therefore, two points to consider in the determination of E 600-esterase in tissue homogenates. First, *p*-nitrophenol produced by the hydrolysis of E 600 might be partially reduced, and secondly, the substrate, E 600, might be depleted by its reduction. These possibilities have been examined and it has been shown that there is no significant loss of *p*-nitrophenol from rat-liver homogenate after incubation for 30 min. and that the same E 600-esterase activity is found whether the determination is carried out with or without 10^{-2} M-KCN. It has been concluded that the method as described is suitable for the determination of E 600-esterase in tissue homogenates.

Table 2. *Inhibition of E 600-esterase by metal inhibitors*

(For both methods of determination of activity, the enzyme was incubated with the inhibitor for 15 min. at 37° before adding the substrate.)

Inhibitor	Molar concentration of inhibitor to produce 50 % inhibition	
	Colorimetric method	Manometric method
Nickel sulphate	$7{\cdot}6 \times 10^{-6}$	$1{\cdot}4 \times 10^{-5}$
Copper sulphate	$8{\cdot}9 \times 10^{-6}$	4×10^{-5}
p-Chloromercuribenzoic acid	$4{\cdot}0 \times 10^{-6}$	$1{\cdot}8 \times 10^{-5}$

The results of an examination of the distribution of E 600-esterase in the rabbit, rat and in various sera are given in Figs. 2–4 respectively. The outstanding feature in the rabbit (Fig. 2) is the high activity of the serum. Such a high activity makes the assessment of tissue activity difficult. For example, unperfused liver hydrolysed 9 mg. *p*-nitrophenol g./30 min. while perfused liver hydrolysed 3 mg. In the rat (Fig. 3) the serum activity is low and the tissue with the highest activity is the liver. Pancreas, brain and submaxillary gland which contain high concentrations of hydrolytic enzymes have no appreciable E 600-esterase activity. Amongst the sera examined (Fig. 4) rabbit serum is the most active, followed by that of ferret and sheep. Every serum tested had a measurable enzyme activity even though it was low in many cases.

Table 3. *Fractionation of rabbit serum by the method of Cohn* et al. (1950) *and by ammonium sulphate*

(For Cohn's method the precipitations were carried out at $-5°$ and centrifuged at $+5°$. Using ammonium sulphate both precipitation and centrifuging were done at $+5°$. The precipitates were dissolved in buffer containing 4×10^{-2} M-cysteine. E 600-esterase was determined colorimetrically, and protein by the colorimetric biuret method of Robinson & Hogden (1940).)

Fraction	Enzyme activity (mg. *p*-nitrophenol/ 100 mg. protein/30 min.)	Total enzyme activity recovered (mg. *p*-nitrophenol/30 min.)	
	Method of Cohn *et al.* (1950)		
I + II + III	59	1150	
IV + V	4·9	204	1354
VI	Nil	Nil	
Original serum	20·7	1332	
	Ammonium sulphate fractionation		
40% saturation (ppt.)	6·9	35	
80% saturation (ppt.)	27·2	147	1003
80% saturation (supernatant)	26·8	821	
Original serum	20·2	1110	

E 600-esterase and other enzymes in rabbit serum. E 600-esterase may be clearly differentiated from the cholinesterases by their differing sensitivities to eserine and to organophosphorus inhibitors. Rabbit serum, which contains the highest E 600-esterase activity of any tissue, also contains a number of interesting enzymes which will hydrolyse atropine, monoacetylmorphine, diacetylmorphine (heroin) and an enzyme distinct from pseudocholinesterase which will hydrolyse benzoylcholine but not acetylcholine. It has been shown that the activity against atropine (Bernheim & Bernheim, 1938; Glick & Glaubach, 1941), monoacetylmorphine (Wright, 1942) and benzoylcholine (Ellis, 1947) is only

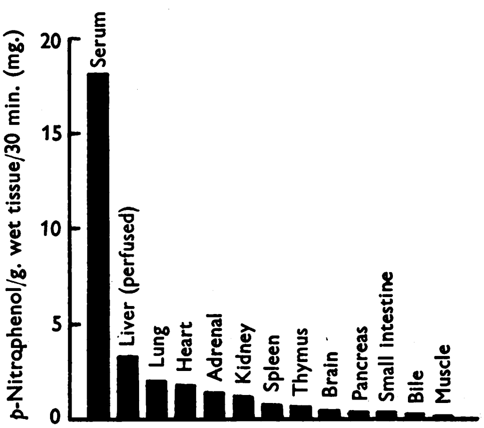

Fig. 2. E 600-esterase activity of tissues of the rabbit. With the exception of liver, which was perfused, tissues were rinsed in saline prior to homogenization with a Folley-type homogenizer in M/15 phosphate buffer, pH 7·6, containing 2% (w/v) gelatin. After suitable dilution with buffer-gelatin solution 0·5 ml. was taken for the determination of activity by the colorimetric method.

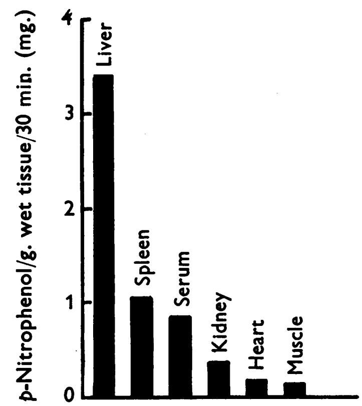

Fig. 3. E 600-esterase activity of rat tissues. Method of determination as given in Fig. 2. The activity of pancreas, brain and submaxillary gland was undetectable.

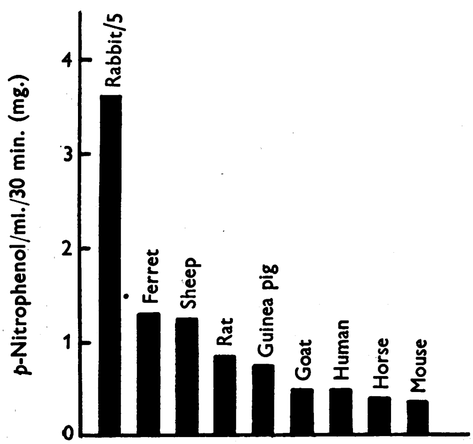

Fig. 4. E 600-esterase activity of sera of various species. Method of determination as given in Fig. 2. The activity of rabbit serum is five times the value plotted.

present in the sera of some but not all rabbits. E 600-esterase has never been absent from the sera of over one hundred rabbits. E 600-esterase may be further differentiated from these enzymes and from procaine esterase (Kisch, Koster & Strauss, 1943) by the fact that they are all inhibited by 10^{-5}M-eserine, procaine esterase (Legge & Durie, 1942; Kisch, 1943), monoacetylmorphine and atropine esterase (Wright, 1941; Glick, 1942) and benzoylcholinesterase (Ellis, 1948). The three latter enzymes are inhibited by tetraethyl pyrophosphate (Ellis, 1948).

E 600-esterase and phosphatase activity. Since E 600 is an ester of phosphoric acid the possibility has been examined that E 600-esterase is identical with acid or alkaline phosphatases. It has been shown (Fig. 2) that rabbit kidney, small intestine and bile which have a high alkaline phosphatase activity are low in E 600-esterase activity. Rabbit

Table 4. *Hydrolysis of E 600 and p-nitrophenyl phosphate by the tissues of the rabbit*

(*p*-Nitrophenol liberated was determined colorimetrically. Sørensen's phosphate buffer, pH 7·6, used for E 600-esterase and a barbitone buffer, pH 7·6, for *p*-nitrophenyl phosphate hydrolysis. 1 unit of enzyme is that amount of enzyme which will liberate 1 mg. of *p*-nitrophenol in 30 min. at 37°.)

Tissue	E 600-esterase (units/ml. or g.)	*p*-Nitrophenyl phosphate hydrolysis (units/ml. or g.)
Serum	24·0	0·02
Bile	Nil	0·04
Kidney* (1)	0·64	3·4
Kidney* (2)	0·13	9·03

* Two different animals.

serum which has a high E 600-esterase activity has a low alkaline phosphatase activity. Spleen, which contains a high acid phosphatase activity, is very low in E 600-esterase. A comparison has been made between the hydrolysis of E 600 and *p*-nitrophenyl phosphate by three tissues of the rabbit. The results in Table 4 show that E 600-esterase cannot be identical with either of the phosphatases.

E 600-esterase and A-esterase

In a previous paper (Aldridge, 1953) it has been demonstrated that there is present in the sera of the rabbit, rat and horse an enzyme (A-esterase) which hydrolyses *p*-nitrophenyl acetate, propionate and butyrate but which is not inhibited by E 600 or similar inhibitors. Since E 600 is a tri-ester of phosphoric acid it is virtually an uncharged molecule and is similar in this respect to *p*-nitrophenyl acetate, propionate and butyrate. Both E 600-esterase and A-esterase are sensitive to nickel, copper and *p*-chloromercuribenzoic acid and neither is inhibited by eserine. The possibility that E 600-esterase and A-esterase are identical has been examined using as enzyme preparations, rabbit, rat and horse sera.

Summation experiments. The substrates *p*-nitrophenyl acetate and E 600 and also *p*-nitrophenyl butyrate and E 600 have been used for these experiments. When the acetate was used, the experiments were carried out using saturated solutions with no substrate in suspension. This procedure was necessary since when a suspension of E 600 was added to a suspension of *p*-nitrophenyl acetate an immediate coagulation of the acetate occurred. It was therefore probable that replacement of the hydrolysed acetate by solution from this coagulum

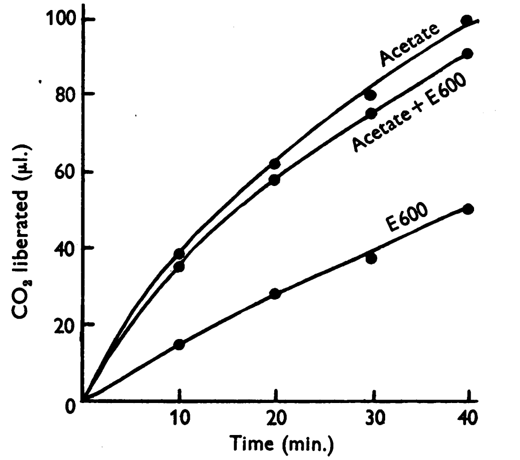

Fig. 5. Summation experiments with rabbit serum using *p*-nitrophenyl acetate and E 600 as substrates. Saturated solutions of substrates in the bicarbonate buffer were used and 0·5 ml. of 100-fold diluted rabbit serum as enzyme. Substrate used shown against each curve.

was slow. The results of these experiments are given in Figs. 5–7, and those using *p*-nitrophenyl butyrate and E 600 are given in Table 5. Both series of experiments are consistent with a competition of the enzyme active centre for both substrates.

Ratio of A-esterase to E 600-esterase activities for different preparations. This ratio has been determined for samples of sera from different animals. The sera of the three species, rabbit, rat and horse have been examined. The results (Table 6) show that this ratio is constant within a species and support the idea that A-esterase is the enzyme which hydrolyses E 600. It is interesting that there is a marked difference between the ratio from one species to another. An enzyme preparation purified by Cohn's method (cf. Table 3, fraction I + II + III) gave the same ratio.

Inhibition of E 600-esterase and A-esterase by diethyl phenyl phosphate. Diethyl phenyl phosphate

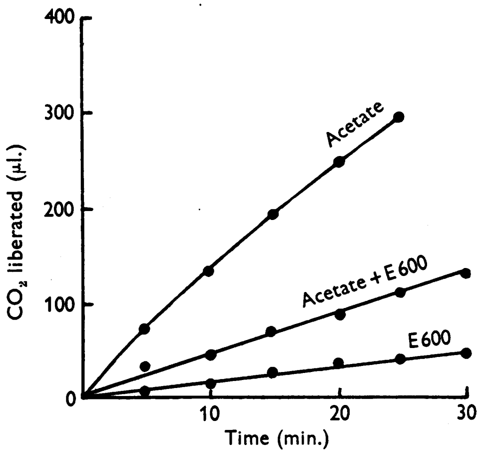

Fig. 6. Summation experiments with horse serum using *p*-nitrophenyl acetate and E 600 as substrates. Saturated solutions of substrates in the bicarbonate buffer were used and 0·5 ml. of fivefold diluted horse serum as enzyme. The substrates used are shown against each curve.

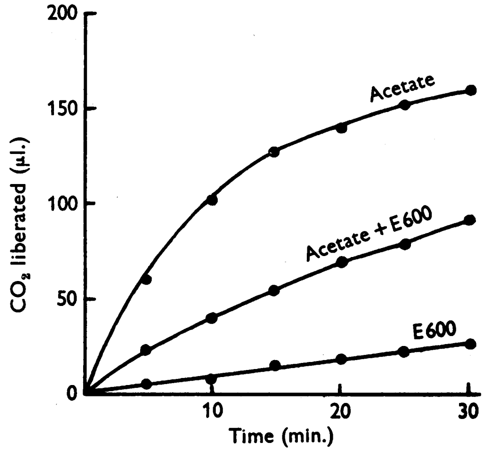

Fig. 7. Summation experiment with rat serum using *p*-nitrophenyl acetate and E 600 as substrate. Saturated solutions of substrates in the bicarbonate buffer were used and 1·0 ml. of fivefold diluted rat serum as enzyme. Substrate used shown against each curve.

Table 5. *Summation experiments using* p-*nitrophenyl butyrate and E* 600

(A-esterase preparation. A suitable dilution of the serum incubated with 5 μg./ml. of E 600 for 30 min. at 37° to remove B-esterase and cholinesterase. A negative sign for (*c*) − (*b*) or (*c*) − (*a*) indicates that with both substrates present the output of CO_2 was less than the greater of the two substrates separately.)

	Enzyme activity (μl. CO_2/ml./min.)				
Serum	*p*-Nitrophenyl butyrate (*a*)	E 600 (*b*)	E 600 + butyrate (*c*)	(*a*) + (*b*)	(*c*) − (*b*) or (*c*) − (*a*)
Rabbit	66	94	72	160	−22
	60	90	67	150	−23
	58	89	65	147	−24
Rat	30	11	15	41	−15
	28	10	15	38	−13
	29	13	18	42	−11
Horse	18	8	16	26	− 2
	20	8	16	28	− 4

is very stable to hydrolysis. Its half life at pH 7·6 at 37° in M/15 phosphate buffer is 8·2 years (Aldridge & Davison, 1952) and it is approximately 250 times more stable than E 600. Since it has a chemical structure very similar to E 600 it is reasonable to assume that it will 'fit' the active centre of E 600-esterase. If E 600-esterase is identical with A-esterase then this compound should also inhibit the hydrolysis of *p*-nitrophenyl acetate. The results in Fig. 8 are consistent with this view. Experiments with A-esterase from rat and horse sera show that in the presence of $2{\cdot}8\times10^{-2}$ M-diethyl phenyl phosphate 65 and 63 % inhibition of activity, respectively, was obtained.

Table 6. *Ratio of A-esterase and E* 600-*esterase activities for the sera of the rabbit, rat and horse*

(A-esterase preparation. B-esterase and cholinesterase activity was prevented by incubation of a suitable dilution of serum with 5 μg./ml. E 600 for 30 min. at 37°. The enzyme activities were determined by the manometric methods. Substrate suspensions were for E 600, 3 mg./ml., and for *p*-nitrophenyl acetate, 4 mg./ml. Enzyme activities are expressed as μl. CO_2/ml./min.)

Serum	No. of samples	Ratio: $\frac{\text{A-esterase}}{\text{E 600-esterase}}$ ± S.D.
Rabbit	10	4·03 ± 0·14
Rat	6	12·2 ± 0·42
Horse	6	18·8 ± 0·81

DISCUSSION

An enzyme has been found which will hydrolyse the tri-ester of phosphoric acid, E 600. This work disproves the statement of Roche (1950) that such esters are not hydrolysed enzymically. It has been demonstrated that by three tests E 600-esterase is identical with an enzyme hydrolysing *p*-nitrophenyl acetate which is not inhibited by E 600 (A-esterase). The sera of the rabbit, rat and horse have been examined and, although E 600-esterase is the same as A-esterase in each species, the enzymes vary from species to species (Table 6). It is becoming clear that enzymes of the same type from different

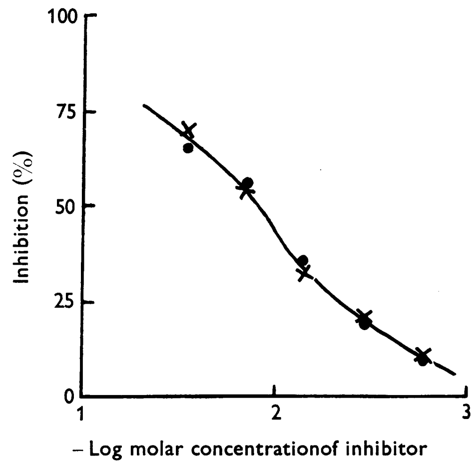

Fig. 8. Inhibition of A-esterase and E 600-esterase of rabbit serum by diethyl phenyl phosphate. A-esterase preparation was a suitable dilution of serum incubated with 5 μg./ml. E 600 for 30 min. at 37°. Saturated solutions of *p*-nitrophenyl acetate and E 600 were used as substrates for A-esterase and E 600-esterase respectively. To these were added varying amounts of a solution saturated with the substrate and diethyl phenyl phosphate. ×, A-esterase; ●, E 600-esterase.

species may have a different spectrum of activities against a number of substrates. This is clear from recent work on cholinesterases from human and horse sera (Mounter & Whittaker, 1950) and from rat heart (Ord & Thompson, 1951). Such differences in proteins from different species are to be expected. Species differences in proteins have been long studied immunologically, and it is well known that not only do the haemoglobins from different species each have their own crystalline form (Reichert & Brown, 1909), but also show functional differences in their affinities for oxygen and carbon monoxide (Anson, Barcroft, Mirsky & Oinuma, 1924). Once species specificity of enzymes is accepted the commonly used method of testing the identity of two activities by comparing the order of the rates of hydrolysis of two substrates by several enzyme preparations from different species, is no longer a valid procedure. On such a procedure Kisch *et al.* (1943) deduced that procaine esterase was not identical with pseudocholinesterase. However, the careful work of Kalow (1952) has shown that these two activities are mediated by the same enzyme. These two conflicting conclusions can be explained by assuming that the ratio of the rates of hydrolysis of procaine and acetylcholine will vary from species to species with the order of activities also varying.

Several enzymes are known to hydrolyse nitrophenyl esters, erythrocyte cholinesterase (Zeller, Fleischer, McNaughton & Schweppe, 1949), human serum cholinesterase (Whittaker, 1951), acetyl esterase from oranges (Jansen, Nutting & Balls, 1948), chymotrypsin (Hartley & Kilby, 1952) and A- and B-esterase of many sera (Aldridge, 1953). Five of the above six enzymes are inhibited by organophosphorus compounds, chymotrypsin (Jansen, Nutting, Jang & Balls, 1949*a*; Jansen, Curl & Balls, 1951; Hartley & Kilby, 1950), acetyl esterase (Jansen *et al.* 1948), B-esterase (Aldridge, 1953) and true and pseudocholinesterase. A-esterase is not inhibited, and in this paper it is shown that it hydrolyses E 600. A consideration of the mechanism of inhibition of enzymes by organophosphorus compounds provides a clue to a possible explanation of this striking difference in behaviour. Jansen *et al.* (1949*a*, *b*) have shown when chymotrypsin is inhibited by di*iso*propyl fluorophosphonate, 1 mol.prop. of hydrofluoric acid is liberated and the di*iso*propyl phosphate radical remains attached to the enzyme. Analogy with this reaction has suggested that the inhibition of other enzymes by such compounds will be by a similar mechanism. This view is substantiated by the fact that when pseudocholinesterase is inhibited by di*iso*propyl fluorophosphonate, phosphorus remains attached to the enzyme (Boursnell & Webb, 1949) and that, for a series of analogues of E 600 of widely varying stabilities to hydrolysis, the more stable the compound is to hydrolysis the less effective it is as an inhibitor (Aldridge & Davison, 1952). The evidence available suggests that the inhibitor behaves as a substrate and is hydrolysed, but that one of the products, the substituted phosphoric acid, remains attached to the enzyme thus preventing its activity. If this be true then all the six enzymes discussed above which can hydrolyse nitrophenyl esters can also hydrolyse E 600, but in only one case (A-esterase) do both products of the hydrolysis pass into the solution leaving the active centre unaltered.

SUMMARY

1. An enzyme has been demonstrated which will hydrolyse diethyl *p*-nitrophenyl phosphate (E 600-esterase).

2. This enzyme is widely distributed in the tissues of the rabbit and the rat and in sera from many different species.

3. Some of the properties of the enzyme in rabbit serum have been examined.

4. By summation experiments, inhibition by diethyl phenyl phosphate, and by the ratio of the activities in different enzyme preparations, it has been shown that E 600-esterase is identical with A-esterase.

5. These enzymes (activities) in the serum of the rabbit, rat and horse have different substrate specificities.

6. The implications of these observations are discussed.

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